Plasma Adrenomedullin Levels in Type 1 Diabetes

Relationship with clinical parameters

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OBJECTIVE — To assess the relationship between plasma adrenomedullin (AM) levels and the presence of microvascular complications in type 1 diabetic patients.

RESEARCH DESIGN AND METHODS — We measured plasma AM and cAMP levels in 103 type 1 diabetic patients (46 without complications, 24 with retinopathy only, 14 with microalbuminuria but normal kidney function, and 19 with renal insufficiency) and 41 matched healthy control subjects.

RESULTS — Patients with renal insufficiency had higher levels of AM and cAMP than all other groups. Patients with only retinopathy showed a trend to have higher levels than patients without complications. There were no differences among all other groups. There was a significant correlation between AM and cAMP in the total diabetic group ($r_s = 0.36$, $P < 0.001$) but not in the control group. In multiple regression analysis, plasma AM demonstrated significant relationships with creatinine clearance ($\beta = -0.31$, $P = 0.004$) and duration of the disease ($\beta = 0.28$, $P = 0.008$).

CONCLUSIONS — Plasma AM and cAMP are increased in type 1 diabetic patients with renal insufficiency. Creatinine clearance (CrClc) and duration of the disease are related to plasma AM levels in these patients.

Human adrenomedullin (AM) is a recently identified 52–amino acid peptide that was originally isolated from an extract of pheochromocytoma tissue (1). This vasodilatory peptide has sequence homology to calcitonin gene–related peptide (1). AM is produced not only in normal adrenal medulla, but also in the lungs, kidneys, and cardiovascular system (heart, endothelial, and vascular smooth muscle cells) (2). The major source of circulating AM is probably the vascular endothelium rather than the adrenal gland (3,4). AM, acting as an autocrine, paracrine, or endocrine vasorelaxing factor, may be important in the regulation of local and systemic vascular tone (5). AM increases intracellular cAMP in vascular smooth muscle cells via its specific receptors, although an endothelium-derived nitric oxide–dependent vasorelaxing mechanism is also involved (5).

Plasma AM levels are increased in different clinical situations, such as essential arterial hypertension (6–8), congestive heart failure (8,9), acute myocardial infarction (10), chronic renal failure (6,8), liver cirrhosis (8), or sepsis (11). The source of AM in these situations is probably the cardiovascular system, as a consequence of endothelial activation. Plasma levels of AM correlate significantly with those of cAMP in hypertension and renal failure (6).

Generalized vasodilatation is observed from diagnosis in type 1 diabetes (12). This vasodilatation is more evident in the afferent arteriole, and it is believed to lead to capillary hypertension, thus contributing to the development of microangiopathy (12). Many different mechanisms seem to be responsible for this phenomenon (12). It has been recently reported that AM is able to induce relaxation in isolated canine retinal arteries (13) and to increase the diameter of both afferent and efferent glomerular arterioles (14). On the other hand, some type 1 diabetic patients develop microalbuminuria. These patients have generalized dysfunction of the vascular endothelium, as evidenced by a high rate of transcapillary albumin escape and raised levels of von Willebrand factor, tissue-type plasminogen activator, etc. (12). In this context, AM could be a new marker of endothelial dysfunction. Finally, some patients progress toward renal insufficiency, a situation in which high plasma AM levels have been reported (6,8).

Hayashi et al. (15) have recently reported that plasma AM levels were increased in a small group of poorly controlled diabetic patients without renal failure. To the best of our knowledge, the potential role of AM in the pathogenesis of diabetic vascular complications has not been studied. The aim of this study was to assess whether plasma AM levels were abnormal in uncomplicated type 1 diabetic patients and in those with different degrees of microangiopathy. We also evaluated the relationship between AM and cAMP levels and other clinical parameters in these patients.

RESEARCH DESIGN AND METHODS

— Table 1 summarizes the clinical details of the healthy control and diabetic groups. There were no differences in age, distribution of sexes, or BMI among the different groups. Diabetic patients were regularly treated in our hospital outpatient clinics and were metabolically stable. They were included consecutively into this cross-sectional study after having given informed consent. The protocol was approved by the Investigation Committee of our institution. Diagnosis of type 1 diabetes was established by onset of the diabetes before 30 years, insulin dependency from diagnosis, and lack

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Abbreviations: AM, adrenomedullin; CrClc, creatinine clearance; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay; TFA, trifluoroacetic acid.

Table 1—*Clinical details*

	Control group	Without complications (group 1)	Only retinopathy (group 2)	Microalbuminuria (group 3)	Renal insufficiency (group 4)
n	41	46	24	14	19
Sex (M/F)	17/24	20/26	10/14	5/9	10/9
Age (years)	37 ± 9	37 ± 5	38 ± 8	37 ± 9	42 ± 11
	36 (25–56)	36 (30–56)	39 (21–54)	38 (22–55)	42 (22–63)
Disease duration (years)	—	9.2 ± 6.7	17.5 ± 8.1	18.5 ± 7.6	20.8 ± 6.9
	—	8 (1–28)	17 (4–31)	18.5 (5–33)	21 (7–34)
Smokers	10	12	7	6	3
BMI (kg/m^2)	23.7 ± 3.3	24.3 ± 2.7	25.1 ± 3.5	25.7 ± 3.1	24.4 ± 3.3
	23 (18–32)	23 (19–32)	25 (18–31)	25 (20–31)	23 (18–30)

Data are *n* or means ± SD and median (range).

of response of C-peptide after glucagon administration. Exclusion criteria were congestive heart failure, liver cirrhosis, chronic obstructive pulmonary disease, asthma, or sepsis. Diabetic patients were classified in four groups: *1)* without any kind of vascular complications, *2)* with retinopathy only, *3)* with microalbuminuria but normal serum creatinine levels, and *4)* with renal insufficiency. Presence of diabetic retinopathy was diagnosed by ophthalmoscopy after pupil dilatation by a specialized ophthalmologist and was scored as nonproliferative or proliferative. Patients were considered hypertensive when blood pressure was ≥140 mmHg for systolic and ≥90 mmHg for diastolic for three consecutive measurements over a period of 4 weeks (16). Microalbuminuria was diagnosed when first morning urine sample albumin-to-creatinine ratio was between 30 and 300 mg/g in two separate specimens, excluding urinary infection (17). Renal insufficiency was diagnosed when serum creatinine levels were persistently >1.3 mg/dl. Patients in groups 1 and 2 were on treatment only with insulin. In group 2, 21 patients had simple retinopathy, and 3 had proliferative retinopathy. None of them had arterial hypertension. In group 3, two patients did not have retinopathy, eight had simple retinopathy, and four had proliferative retinopathy. Three patients had arterial hypertension. Ten had been on treatment with ACE inhibitors for at least 6 months. Some were also on treatment with diuretics or hypolipemic drugs. Patients in groups 2 and 3 did not present a previous history of cardiac angina, intermittent claudication, or myocardial or cerebral infarction, and resting electrocardiogram was normal. In group 4, 1 patient had simple retinopathy, and 17 had proliferative retinopathy. Three patients were on hemodialysis. Eight had symptomatic macroangiopathy. Thirteen patients were on treatment with ACE inhibitors, seven with diuretics, one with calcium channel inhibitors, and four with statins.

Fasting venous blood samples were obtained after 30 min of supine rest from an antecubital vein before the morning injection of insulin. Glucose, creatinine, and cholesterol were measured by automated methods on a Technicon Dax (Technicon Instruments, Tarrytown, NY), using the reagents supplied by Boehringer-Mannheim (Mannheim, Germany). CrClc was calculated with the Cockroft-Gault formula (18). HbA_{1c} was determined by automated high-performance liquid chromatography (HPLC) (Diamat; BioRad, Munich, Germany). Urine albumin excretion rate was quantified by immunoturbidimetry (Behring Nephelometer Analyzer II; Behring Diagnostics, Marburg, Germany).

AM was measured using the kit supplied by Peninsula Lab (Belmont, CA), following the manufacturer's instructions. Blood was obtained using a chilled syringe and immediately transferred into a chilled polypropylene tube containing EDTA (1 mg/ml of blood) and aprotinin (500 kU/ml of blood). Blood was centrifuged at 1,600*g* for 15 min at 4°C. Plasma was immediately frozen and stored at −80°C until assayed. Plasma AM concentration was measured by a specific radioimmunoassay (RIA) after extraction through the Sep-Pak C–18 column supplied by the manufacturer. First, 1 ml plasma was acidified with 1 ml of 1% trifluoroacetic acid in distilled water (TFA) (HPLC grade, Merck, Darmstadt, Germany) and centrifuged at 8,000*g* for 20 min at 4°C. Columns containing 200 mg of C–18 were equilibrated by washing with 1 ml of 60% acetonitrile (Merck) in 1% TFA (once) followed by 3 ml of 1% TFA (three times). Acidified plasma was added to the pretreated columns without applying pressure. The columns were slowly washed with 3 ml of TFA (twice), and the wash was discarded. The peptide was eluted very slowly with 3 ml of 60% acetonitrile in 1% TFA, and the eluant was collected in a polypropylene tube. Eluant was evaporated using a Speed-Vac Concentrator SVC 100 H (Savant Instruments, Farmingdale, NY). The recovery of AM after extraction was determined by adding AM to plasma before extraction and was 78%. On the next day, the residue was dissolved in 250 µl of RIA buffer, vortexed, and centrifuged at 8,000*g*, and two aliquots of 100 µl were used to assay. Standard peptide (1.28 µg) was reconstituted with 1 ml of RIA buffer, and successive dilutions were made to achieve an effective range of the standard curve between 10 and 1,280 pg/ml. Rabbit anti-AM antiserum, ^{125}I-AM, and precipitating agents were prepared and used according the manufacturer's instructions. Radioactivity was measured using an LKB Wallac 1272 CliniGamma Counter (Turku, Finland). The results were automatically calculated using logit B/Bo vs. ln concentration, and the final results were divided by four (concentration factor). This assay does not show any cross-reactivity with human AM (13–52), human CGPR, endothelin–1, atrial natriuretic peptide, brain natriuretic peptide, or C-type natriuretic peptide. Sensitivity was 5 pg/ml. Interassay and intra-assay coefficients of variation were 15 and 10%, respectively. To diminish variability, in each assay we matched a similar number of samples of the different groups.

Plasmatic cAMP levels were measured by RIA (Incstar, Stillwater, MN) after preliminary acetylation of the samples.

Table 2—*Biochemical parameters*

	Control group	Without complications (group 1)	Only retinopathy (group 2)	Microalbuminuria (group 3)	Renal insufficiency (group 4)
Glucose (mg/dl)	90 ± 12	167 ± 80	168 ± 88	192 ± 97	188 ± 138
	89 (68–119)	148 (40–360)	158 (55–359)	196 (47–365)	123 (46–578)
HbA_{1c} (%)	5.3 ± 0.3	8.2 ± 1.4	8.2 ± 1.1	9.4 ± 1.3	9.4 ± 1.6
	5.3 (4.8–6.3)	8 (5.1–1.3)	8 (6.7–11.6)	9.9 (7.3–11.1)	9.1 (6.6–13.3)
Creatinine (mg/dl)	0.99 ± 0.11	1.01 ± 0.12	1.00 ± 0.14	1.02 ± 0.12	2.56 ± 1.56
	1 (0.8–1.2)	1 (0.8–1.2)	1 (0.8–1.2)	1.1 (0.8–1.2)	1.7 (1.3–7.1)
CrClc ($ml \cdot min^{-1} \cdot m^{-2}$)	85.1 ± 10.5	86.6 ± 10.4	86.2 ± 14.3	84.7 ± 11.2	41.2 ± 17.5
	85.6 (63.6–117.2)	87.5 (63.7–112.6)	82.3 (64.6–113.6)	87.4 (58.1–100.1)	46.2 (8.7–66.5)
Microalbuminuria	—	8.9 ± 6.9	7.5 ± 3.7	133.3 ± 97	—
(mg:g creatinine)	—	6.3 (0.6–27.3)	6.5 (3.1–18.2)	106.3 (31–305)	—
Proteinuria (g/24 h)	—	—	—	—	2.17 ± 1.52
	—	—	—	—	2 (0.2–5)
Cholesterol (mg/dl)	189 ± 37	175 ± 28	193 ± 40	180 ± 48	205 ± 43
	188 (120–246)	172 (126–246)	194 (131–291)	171 (84–290)	197 (156–313)
AM (pg/ml)	82.7 ± 40.8	78.1 ± 28 ± 1	101.4 ± 40	96.4 ± 44.5	235.7 ± 138.8
	93 (15–172)	77 (16–155)	91.5 (48–178)	87.5 (48–192)	188 (81–555)
cAMP (nmol/l)	9.4 ± 1.7	8.8 ± 2.0	9.4 ± 2.6	9.8 ± 2.3	12.2 ± 3.1
	9 (6.5–14)	9 (5–13.5)	9.5 (6.5–18)	9.3 (7.5–16.5)	11 (9–20.6)

Data are means ± SD and median (range).

All values are given as means ± SD and medians (range). When comparing more than two nonnormally distributed variables, the Kruskal-Wallis test was used. If differences were found, the Mann-Whitney test with Bonferroni adjustment was used for comparisons between two groups. For normally distributed variables, analysis of variance was performed. If differences were found, Scheffe's test was used for comparison between two groups. Correlation between AM and cAMP levels was done using Spearman's coefficient, since they were nonnormally distributed. When considering possible associations to AM levels, multiple regression analysis (stepwise selection) was used after logarithmic transformation of the nonnormally distributed variables. The following variables were studied: age, BMI, duration of diabetes, blood glucose, HbA_{1c}, cholesterol, albumin excretion rate, and calculated CrClc.

All calculations were made with Statistical Package for the Social Sciences software (SPSS, Chicago). A P value <0.05 was considered statistically significant.

RESULTS — Table 2 shows the biochemical parameters of the control and diabetic groups. As expected, glucose and HbA_{1c} were higher than normal in all diabetic groups. Group 4 patients (with renal insufficiency) had higher levels of AM than all other groups ($P < 0.001$). The levels of AM in the three patients on hemodialysis were 160, 525, and 555 pg/ml. Group 2 patients (with only retinopathy) also showed a trend toward higher levels of AM than group 1 ($P = 0.039$), but because of the exigency of Bonferroni's adjustment, the comparison did not reach statistical significance. Plasma cAMP levels in group 4 were higher than in all other groups ($P < 0.007$), but no differences were found among the other groups. There was a significant correlation between AM and cAMP in the total diabetic group (Spearman's r coefficient 0.36, $P < 0.001$) (Fig. 1) but not in the control group. No correlation was found between plasma AM and blood pressure in any group. In multiple regression analysis, plasma AM demonstrated a significant inverse relationship with CrClc (coefficient β −0.31, $P = 0.004$) and a positive relationship with duration of the disease (coefficient β 0.28, $P = 0.008$), according to the formula log AM = 2.17 − (0.004 × CrClc) + (0.007 × duration).

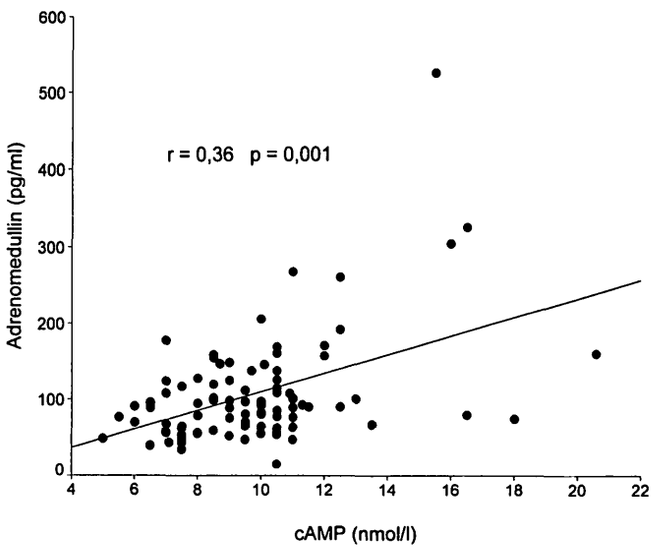

Figure 1—*Correlation between AM and cAMP in the entire group of type 1 diabetic patients.*

CONCLUSIONS — The levels of plasma AM in our control group are higher than those reported previously by other authors (6–10), but they are similar to those reported by another European group (19), and even higher levels than ours have been reported in normal women, using the same assay (20). Our data demonstrate that plasma AM levels are clearly increased in type 1 diabetic patients with kidney failure, as reported in other causes of renal insufficiency (6,8). Furthermore, we have found a significant inverse correlation with CrClc, suggesting that as kidney function deteriorates, AM increases proportionally. The increase in AM levels could be the result of a decreased clearance of the peptide by the kidney, although most of the circulating AM seems to be metabolized in the pulmonary circulation (3,6). Alternatively, high AM levels may reflect endothelial activation, since this peptide may be involved in protective mechanisms against blood pressure elevation and/or body fluid volume expansion (6). The positive correlation in diabetic patients between AM and cAMP, a second messenger involved in the regulation of the circulatory system, supports the hypothesis that AM plays an active counterregulatory role, preventing excessive vasoconstriction, promoting natriuresis and, possibly, inhibiting excessive platelet aggregation (21). The trend to higher levels in patients with retinopathy also raises the intriguing possibility that AM may be somehow involved in the pathogenesis of diabetic retinopathy, since retinal arteries are known targets of this peptide (13). It has been recently demonstrated that AM acts as an apoptosis survival factor for endothelial cells (22), so, it may play a protective role in the diabetic retinal vessels. On the other hand, it could also induce vasodilatation of these vessels. The normal levels of AM in patients with microalbuminuria were somewhat unexpected. It is possible that the treatment with ACE inhibitors may have normalized previously elevated AM levels, since these drugs improve endothelial function in these patients (23). It can also be speculated that ACE inhibition itself modifies plasma AM levels. Alternatively, AM could have been lost in urine. Further studies with untreated microalbuminuric patients and/or measuring urine AM excretion are needed to test these hypotheses. The normal levels in patients without complications argue against a primary role of AM in the initial generalized vasodilatation induced by diabetes, although a local paracrine effect cannot be excluded. The relationship with duration of disease suggests that the elevation of AM levels is a late phenomenon due to endothelial dysfunction. In any case, it seems that AM levels are abnormal in type 1 diabetic patients with vascular complications. Similar preliminary results have been recently reported by Japanese investigators in type 2 diabetic patients (24).

Finally, since AM is produced also in the adrenal medulla, it could be considered that AM levels in some diabetic patients could have been raised in response to hypoglycemia, but careful studies inducing hypoglycemia in normal subjects did not find any response in circulating AM levels, suggesting that AM is not involved in the hypoglycemia-induced counterregulatory hormone response (19). Although our study was not aimed to test this point, we did not find either differences in AM levels in hypoglycemic versus hyperglycemic matched patients or a relationship between plasma glucose or HbA_{1c} and AM levels, suggesting that metabolic control, as assessed by these parameters, does not influence AM levels. This point needs clarification, since Hayashi et al. (15) reported that AM mRNA level was increased in the vascular smooth muscle cells cultured in high-glucose medium.

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